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Methods

Case study of non-lethal sampling for plant-pollinator networks via barcoding and metabarcoding on bumble bees in Germany

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Abstract

1. Insect decline is threatening ecosystem stability, making information about foraging preferences of pollinators a vital piece of information to acquire. A powerful emerging tool to study pollinator foraging behaviour is pollen-metabarcoding. This usually involves lethal sampling of insects.
2. Here, we propose a new, non-lethal way of sampling DNA for the analysis of pollen loads of bumble bees as well as the pollinator. The new methodology does not significantly harm the insect and is easy to implement in a wide range of study designs. The tool is cheap and easy to acquire, can easily be used in the field and has the potential to become a powerful tool in studying plant-pollinator interactions.
3. To test its feasibility, plant-pollinator networks were analysed using metabarcoding of the ITS2 region. Plants flowering at the time of collection were also recorded as a reference comparison.
4. Bumble bees with ambiguous morphology were additionally identified, based on COI barcoding.
5. With the workflow developed here, it is possible to gain knowledge about plants and their pollinators in a non-lethal way without reducing population sizes. This makes this method particularly suitable for endangered and protected species.

Key words: Cytochrome c oxidase I (COI), endangered species, internal transcribed spacer (ITS), pollination, species diversity, twinned pollinator-pollen analysis

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Introduction

The ongoing decline of insect populations, both in terms of species numbers (Sánchez-Bayo and Wyckhuys 2019) and biomass (Hallmann et al. 2017), is concerning. This decline heavily affects pollinators, but it also has significant consequences for various plant species they pollinate (Kluser and Peduzzi 2007; Althaus et al. 2021). Cascading effects of this loss can potentially destabilise ecosystems, leading to the collapse of pollination networks and jeopardising overall ecosystem stability (Lever et al. 2014; Rhodes 2018; Dicks et al. 2021). Amongst these vital pollinators, bumble bees (genus *Bombus*) stand out. Social bumble bees play a significant role by collecting nectar from plants and gathering pollen to nourish their offspring (Goulson 2010). They have unique characteristics, including adaptation to colder temperatures, enabling them to pollinate

earlier as well as later in the year (Woodard 2017). Additionally, bumble bees possess the ability for buzz-pollination, a skill which is crucial for pollinating plants in the nightshade family Solanaceae (Pritchard and Vallejo-Marín 2020). However, the precise foraging behaviour of bumble bees remains poorly understood. Though considered polylectic (Woodard 2017), variations in foraging and dietary preferences amongst species (Laverty and Plowright 1988), as well as individuals of the same species (Ruedenauer et al. 2016), have been reported. A better understanding is essential for the conservation of threatened species.

A promising tool for bridging this knowledge gap is pollen metabarcoding, a method to genetically analyse the pollen load of insects and identify the plant species they visit (Bell et al. 2022). However, until now, this method typically requires collecting and killing of insects (Bell et al. 2017). This also allows for genetic identification of bumble bees via DNA barcoding by using insect tissues, as certain species share similar morphology and can only be accurately distinguished by genetic means (Murray et al. 2008). Criticisms were raised regarding lethal collection of insect specimens (Miller et al. 2022), suggesting that this practice might further accelerate the decline of target species. Proposals were made to collect bee specimens only at specific times in the colony life cycle when the loss of workers is less detrimental (Camilo 2022). However, it is crucial to obtain information about foraging behaviour during all life stages. A twinned approach of combining pollen metabarcoding and pollinator barcoding proved to be very promising (Ronca et al. 2023), but still depends on lethal sampling.

We here present a cost-effective, easy to implement, non-lethal alternative for the collection of genetic material from bumble bee pollen and insect tissue. The method ensures that the foraging ability of bumble bees is not significantly impaired. Using a sample dataset of bumble bees collected in northern Germany and the pollen adhering to them, we demonstrate the successful analysis of bumble bee identification via barcoding, as well as pollen analysis via metabarcoding. Plant-pollinator networks are verified by observations of flowering phenology during insect collection.

Methods

Sampling

Sampling took place on 13 June 2023 between 11:00 a.m. and 3:00 p.m. in the nature protected area Cuxhavener Küstenheide (WDPA-ID: 329318) in northern Germany, with permission from the respective nature conservation authority.

A vegetation survey was conducted along a 500 m north-west to south-east transect, recording all flowering plants within a proximity of 30 m. Bumble bees were captured along the transect using a queen-marker-cage (Fig. 1A). This device was originally designed for beekeepers for tagging honey bee queens (e.g. Imgut®, article number 4756). It comprises a tube with a coarse mesh at one end and a punch at the other, which was covered with sterile cling film before each sampling. Insects can be gently immobilised with this device (Fig. 1C). Pollen was collected from the corbicula of immobilised bumble bees using a fine art brush through the mesh. Cuticle scissors were then used to clip the tarsus from one middle leg of the insect (Fig. 1B). This tarsal-clipping of a middle leg does not affect the productivity of bumble bees (Holehouse et al. 2003), but provides sufficient material for

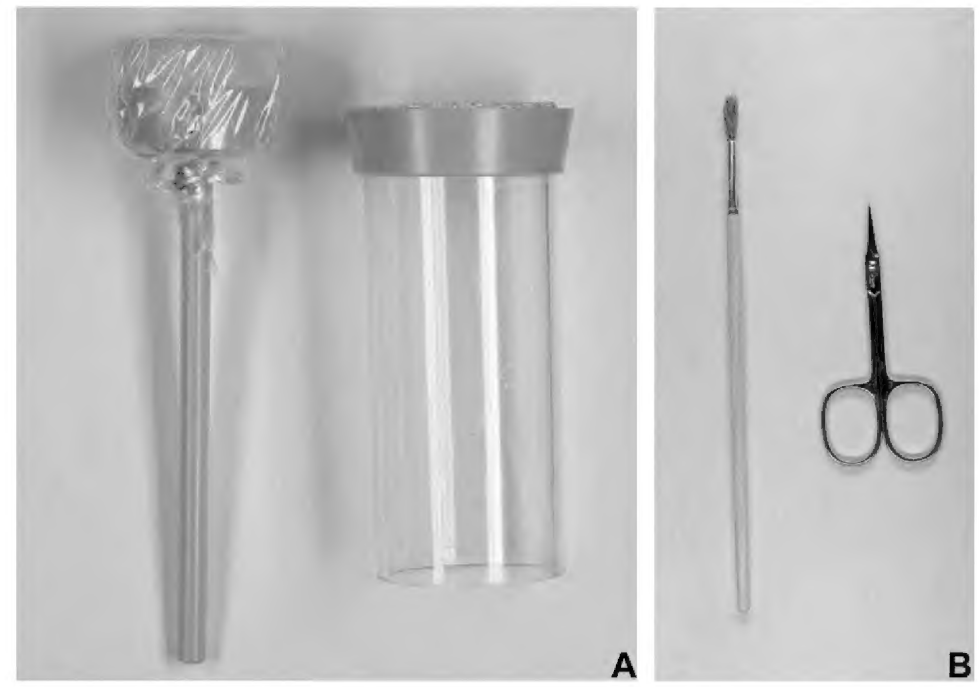

Figure 1. Equipment for non-lethal insect sampling in the field **A** queen-marker-cage for immobilising insects **B** cuticle scissors and fine art brush **C** captured bumble bee in the queen-marker-cage (top), displaying the mesh's large holes through which the equipment can be manoeuvred to dislodge the pollen as well as remove the tarsus.

the genetic analysis. To free the insect, the mesh was cautiously opened. After the insect was released, the punch was lowered to the brim of the tube and the cling film was folded over the genetic material. This wrapped cling film was then placed into a 2 ml centrifuge tube until further processing in the laboratory. To prevent cross-contamination, all equipment, including queen-marker-cages, underwent a rigorous cleansing process with 10% bleach solution. Subsequently, the equipment was rinsed with 70% ethanol and dried off, before reuse in the field.

In total, 21 samples containing both the tarsus as well as the pollen of the insect were collected.

Molecular laboratory work

Laboratory work for plant metabarcoding was carried out in a controlled clean room environment to minimise contamination. Quality control measures comprised controlled airflow, air filtration systems and UV light cleaning. Using sterilised tweezers, the tarsus was removed from the tube and the pollen was scraped from the cling film and transferred to a fresh 1.5 ml centrifuge tube using stainless-steel laboratory spatulas. Homogenisation of the pollen was achieved by bead milling the pollen samples for 2.5 minutes at 30 Hz (Retsch® MM400). Plant DNA extraction was performed using a modified protocol of the NucleoMag DNA extraction kit (Macherey-Nagel, REF744400.4; Kolter et al. (2023)) and included three extraction blanks.

PCR targeting the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA followed Kolter and Gemeinholzer (2021a) and used the primers ITS-3p62plF1 and ITS-4T5unR1 (See Suppl. material 1). The reaction volume was 12.5 µl and used 1 µl template DNA. The final concentration of the PCR mixture consisted of 5% trehalose, 1 × reaction buffer, 5% DMSO, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.24 mg/ml BSA, 0.1 µM of each primer and 0.025 U/µl Platinum Taq (Invitrogen™). The PCR cycling conditions comprised an initial denaturation at 95 °C for 3 minutes, followed by 30 cycles: denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds and elongation at 72 °C for 45 seconds. The final elongation step was conducted at 72 °C for 6 minutes and the samples were stored at 8 °C. Three PCR negative controls were added. Two positive controls were included, consisting of five plant DNA isolates from exotic plants not expected to be present at the sample location (*Clerodendrum thomsoniae*, *Coffea arabica*, *Euphorbia milii*, *Sophora* sp., *Spathiphyllum wallisii*). Three PCR replicates per sample/control were conducted and subsequently pooled. Sequencing as well as library preparation were carried out by LGC-Genomics (Berlin/Germany). At LGC Genomics, additional amplification was performed in a 20 µl reaction volume using 10 µl of 2 × MyTaq HS Red Mix (Bioline GmbH, Luckenwalde, Germany) and standard i7- and i5-sequencing adaptors. The PCR cycling conditions comprised an initial denaturation at 96 °C for 1 minute, followed by 10 cycles of denaturation at 96 °C for 15 seconds, annealing at 58 °C for 30 seconds and elongation at 70 °C for 90 seconds. A final elongation step was conducted at 70 °C for 2 minutes and the samples were stored at 8 °C. Sequencing was done on an Illumina MiSeq (Illumina, Inc., CA, USA) using V3 Chemistry (2 × 300 bp).

COI-barcoding analysis for bumble bee identification was performed in a standard molecular lab environment. For the DNA extraction, the DNeasy® Blood & Tissue Kit (Quiagen, ID: 69506) was used with modifications (See Suppl. material 2). The LCO1490 and HCO2189 primers (Folmer et al. 1994) were used to target the COI gene in a 20 µl reaction mixture with 2 µl template DNA. The final concentration of the PCR mixture consisted of 1 × reaction buffer, 0.2 Mm dNTPs, 2.25 µM of each primer, 0.25 mg/ml BSA and 0.4 U/µl Taq polymerase. The PCR cycling conditions comprised an initial denaturation at 95 °C for 3 minutes, followed by 35 cycles: denaturation at 95 °C for 30 seconds, annealing at 45 °C for 30 seconds and elongation at 72 °C for 45 seconds. The final elongation step was conducted at 72 °C for 6 minutes and the samples were stored at 8 °C. Further details can be found in Suppl. material 1. Positive and negative controls were added to each run, but were only used to evaluate the successful PCR on a 1.5% agarose gel and are not included in the subsequent sequencing analysis. Sanger sequencing was carried out by Eurofins Genomics (Luxembourg City/Luxembourg).

Data analysis

Raw data from Sanger sequencing for the bumble bees underwent editing using MEGA X (Kumar et al. 2018). Taxonomic assignments were performed using BLAST (Altschul et al. 1990) in NCBI against the Core Nucleotide database (Core_nt) on 8 January 2024.

Raw Illumina MiSeq reads of pollen samples were analysed via a bioinformatic pipeline using APSCALE (Buchner et al. 2022), VSEARCH (Rognes et al. 2016), cutadapt (Martin 2011), LULU (Frøslev et al. 2017), SINTAX (Edgar 2016) and R (See Suppl. material 3). APSCALE was used for paired-end merging, primer trimming, quality filtering and denoising (see Suppl. material 4). A custom reference database was constructed, containing 583,533 vouchered plant and fungal ITS2 sequences obtained from GenBank (NCBI, data downloaded on 20.08.2023; Sayers et al. (2021)). The search queries "ITS", "internal transcribed spacer", "voucher", "Streptophyta" and "Fungi" were used and results were processed with the R package taxonomizr (Sherrill-Mix 2023). SINTAX was then used against this reference database to assign taxonomy to the Exact Sequence Variants (ESVs).

The negative controls yielded a total of 9, 46 and 1 reads for the extraction blanks and 15, 7 and 4 reads for the PCR blanks. Due to the low number of reads, those were discarded from the analysis. The positive controls successfully retrieved the expected plant species and were also discarded.

Manuscript preparation

During the preparation of this work, the authors used AI-assisted technology, including DeepL Write and ChatGPT (v.4), to improve language and readability. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Results

In this study, 12 out of 21 investigated bumble bee individuals could unambiguously be identified in the field based on their morphology (1 *B. hypnorum*, 8 *B. lapidarius* and 3 *B. pascuorum*). Nine bumble bees exhibited ambiguous morphological characteristics, identified as 4 *B. lucorum* agg. and 4 *Bombus* sp. Subsequent COI barcoding of these ambiguous individuals revealed 2 *B. cryptarum*, 4 *B. pascuorum* and 3 *B. terrestris*, resulting in the successful identification of all 21 bumble bee individuals, in total belonging to five distinct species (2 *B. cryptarum*, 1 *B. hypnorum*, 8 *B. lapidarius*, 7 *B. pascuorum* and 3 *B. terrestris*). The raw sequence data have been deposited in GenBank and the accession numbers, as well as a full list of species, can be found in Suppl. material 5.

Pollen metabarcoding generated 642,151 raw sequences, which have been deposited in the Sequence Read Archive under accession number PRJNA1120766. Quality-filtered sequences were cross-referenced against the ITS2 database. The read table, including both positive and negative controls used for quality control (but not for the final analysis), is provided in Suppl. material 6. This process resulted in an unambiguous identification of 18 plant species (Fig. 2). However, four ESVs could only be identified to genus level (*Lotus* sp., *Ranunculus* sp., *Rosa* sp. and *Trifolium* sp.). *Trifolium* was the most commonly detected genus, with *T. repens* found on all 21 bumble bees. In contrast, *Jasione montana*, *Lotus* spec., *Prunus persica*, *Ranunculus* spec., *Ranunculus sardous*, *Rubus caesius* and *Rumex acetosella* were detected on only one bumble bee individual each.

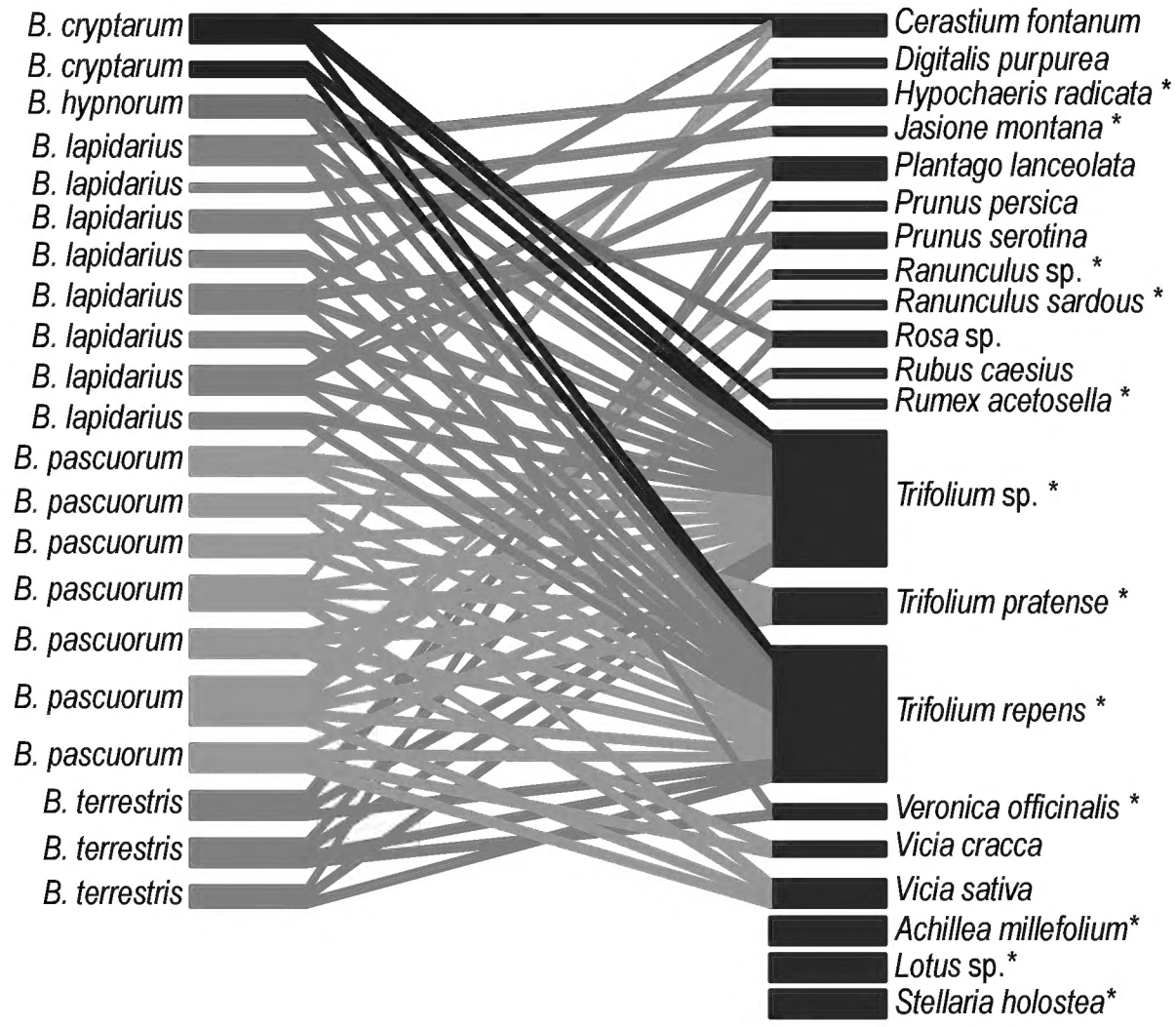

Figure 2. Plant-pollinator network of bumble bees collected in Cuxhavener Küstenheide. Interactions revealed by metabarcoding of pollen of 21 bumble bees and barcoding of insect tissue in a nature conservation area in northern Germany on 13.06.2022. Detected plant species supported by phenology screening are marked with an asterisk.

The vegetation survey yielded 17 different plant species, 13 of which were also detected by pollen metabarcoding. *Achillea millefolium*, *Galium saxatile*, *Hieracium pilosella* and *Stellaria graminea* were flowering, but did not show up in the metabarcoding analysis. *Prunus persica*, *Prunus serotina*, *Rosa* sp. and *Rubus caesius* were found in the pollen DNA analysis, but were not detected on the sampling site. All plant species recorded through either the phenology survey or the pollen metabarcoding analysis are likely to occur on site, except *Prunus persica* (NLWKN 2006; GBIF Occurrence Download from 27 March 2024, https://doi.org/10.15468/dl.9sasyx).

Bumble bee individuals carried pollen from two up to seven different plant species, resulting in a plant-pollinator network with 28 interactions (Fig. 2). The most prominent plant genera were *Trifolium* and *Vicia*. All bumble bee individuals, regardless of species, were found to carry pollen of *Trifolium* sp. and *Trifolium repens*. Pollen belonging to the genus *Vicia*, as well as pollen of *Trifolium pratense*, was exclusively found on *B. pascuorum*. Pollen of *Jasione montana*, *Lotus* sp. and *Hypochaeris radicata* was only found on *B. lapidarius*.

B. cryptarum carried pollen of four different plant species (*Cerastium fontanum*, *Rumex acetosella*, *Trifolium* sp. and *Trifolium pratense*). *B. hypnorum* carried pollen from three plant species (*Rosa* sp., *Trifolium* sp. and *Trifolium repens*). The *B. lapidarius* specimens carried pollen from nine different plant species (*Cerastium fontanum*, *Hypochaeris radicata*, *Jasione montana*, *Plantago lanceolata*, *Prunus persica*, *Prunus serotina*, *Trifolium* sp., *Trifolium repens* and *Veronica officinalis*). Similarly, nine plant species were detected on *B. pascuorum* (*Cerastium fontanum*, *Ranunculus* sp., *Ranunculus sardous*, *Rubus caesius*, *Trifolium* sp., *Trifolium pratense*, *Trifolium repens*, *Vicia cracca*

and *Vicia sativa*). Finally, the *B. terrestris* individuals carried pollen from seven different plant species (*Plantago lanceolata*, *Prunus persica*, *Prunus serotina*, *Rosa* sp., *Trifolium* sp., *Trifolium repens* and *Veronica officinalis*).

Discussion

The new, cost-effective, easy-to-perform and non-lethal alternative for obtaining DNA material from bumble bee pollen and insect tissue proved to be successful.

By conducting pollen metabarcoding, supported by phenological studies and non-lethal insect barcoding in addition to morphological identification, we could deduce plant-insect relationships comparable to earlier studies on the same site (Kolter et al. 2023) and plant-pollinator networks in general (Memmott 1999; Dicks et al. 2002). As previously suggested, morphological identification of bumble bee species, particularly within the *B. lucorum* complex, proved challenging. However, the COI barcode region could resolve these uncertainties as well as complement correct *Bombus*-species level identification (Carolan et al. 2012; Williams et al. 2012; Ronca et al. 2023). The non-lethal sampling strategy, presented here, likely does not harm the insect population, as we observed bumble bees gathering pollen that lacked the mid-tarsus, indicating prior sampling. This echoes findings by Holehouse et al. (2003), suggesting minimal impairment of the insects foraging capabilities. The identified bumble bee species in our sampling align with those found earlier on this site (Sprichardt 2011). While *B. cryptarum* was observed in 2007, it was absent in 2009 (Sprichardt 2011). Our study re-affirms the presence of this species at this site again in 2022.

Pollen metabarcoding successfully provided a species list of plants visited by bumble bees. For plant pollen species level identification, conservative filtering is recommended (Tommasi et al. 2021). We chose a 1% cut-off in accordance with Peel et al. (2019), but if false positives pose a significant risk, a more conservative approach, such as Receiver Operator Characteristics (ROC), should be considered (Serrao et al. 2018). Accurate species level identification is highly dependent on comprehensive reference databases (Kolter and Gemeinholzer 2021b; Keck et al. 2023). Here, we opted for an approach using publicly available sequences, which might have led to some plants being identified only to genus level. A database using regional plants could enhance the resolution.

Although four plants detected in pollen metabarcoding were absent in the flowering vegetation survey, this discrepancy likely stems from the small size of the nature protected area (< 800 m^2) and the known foraging radius of bumble bees, surpassing the surveyed area (Westphal et al. 2006). All plants detected are common in the broader region, except *Prunus persica*, a popular ornamental garden plant. Its presence in our analysis likely stems from such planted trees in the nearby gardens.

With the workflow developed here, it is possible to gain knowledge about plants and their pollinators. The non-lethal sampling approach is not reducing bumble bees' population sizes, but allows for close examination of endangered bumble bees and their plant visits at different times throughout the colony's life cycle. This can mitigate the potential decline of pollinators, a goal also formulated by the IPBES report (Potts et al. 2016). With minor modifications, the method presented here could also be adapted to smaller pollinators, further expanding its applicability.

Conclusion

In this study, we successfully demonstrate a cost-effective, easy-to-perform and non-lethal method for obtaining DNA material from bumble bee pollen and insect tissue. By combining pollen metabarcoding and insect barcoding, we constructed plant-pollinator networks that align with previous research and the phenology on the collection day.

Pollen metabarcoding retrieved a comprehensive species list of plants visited by bumble bees, very similar to the vegetation survey and consistent with the occurrence data for the area. Employing barcoding for insect identification resulted in delimitation of *B. cryptarum* underscoring the necessity of genetic identification of bumble bees.

Our non-lethal sampling approach offers a valuable tool for studying plant-pollinator interactions without reducing bumble bee populations. It allows for the monitoring of endangered species and their foraging habits throughout the entire colony's life cycle, contributing valuable information to efforts aimed at mitigating pollinator decline. Future research could adapt this method for smaller pollinators, broadening its applicability and impact in the field of pollination ecology.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Alexander Edwards and Birgit Gemeinholzer conceived the ideas and designed methodology; Alexander Edwards collected and analysed the data; Alexander Edwards wrote the manuscript with feedback and editing from Birgit Gemeinholzer. All authors contributed critically to the drafts and gave final approval for publication.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information and will be made available in public repositories.

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Supplementary material 1

PCR protocols

Author: Alexander Edwards
Data type: pdf
Explanation note: PCR protocols for both plant pollen and insect tarsi.

Link: https://doi.org/10.3897/mbmg.9.141904.suppl1

Supplementary material 2

Tarsi DNA extraction

Author: Alexander Edwards
Data type: pdf
Explanation note: Modified DNA extraction protocol for use on small tissue samples, such as bumble bee tarsi.

Link: https://doi.org/10.3897/mbmg.9.141904.suppl2

Supplementary material 3

Data analysis

Author: Alexander Edwards
Data type: pdf
Explanation note: The bioinformatic script used to analyze the raw metabarcoding sequences and construct plant-pollinator networks.

Link: https://doi.org/10.3897/mbmg.9.141904.suppl3

Supplementary material 4

APSCALE Settings

Author: Alexander Edwards

Data type: xlsx

Explanation note: Settings file used with APSCALE.

Link: https://doi.org/10.3897/mbmg.9.141904.suppl4

Supplementary material 5

Bumble bee species list

Author: Alexander Edwards

Data type: xlsx

Explanation note: Species list of all bumble bees collected. For morphologically ambiguous specimens, DNA barcoding was conducted, and the corresponding accession numbers are provided.

Link: https://doi.org/10.3897/mbmg.9.141904.suppl5

Supplementary material 6

Read table

Author: Alexander Edwards

Data type: xlsx

Explanation note: Read table containing quality-filtered ITS2 sequences obtained from pollen samples, including corresponding taxonomic assignments for each Exact Sequence Variant (ESV).

Link: https://doi.org/10.3897/mbmg.9.141904.suppl6